Berlin, Jan 17th, 2020

Diagnostic detection of 2019-nCoV by real-time RT-PCR

-Protocol and preliminary evaluation as of Jan 17, 2020-

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Users looking for a workflow protocol consult the last three pages of this document

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Positive control material is available from Charité, Berlin, via EVAg (https://www.european-virus-archive.com/).

This is document Version 2.
Changes against Version 1 (Jan 13, 2019): Workflow protocols included, N gene assay removed, data for single probe versions of RdRp assay added; information on availability of controls updated.

We acknowledge the originators of sequences in GISAID (www.gisaid.org): National Institute for Viral Disease Control and Prevention, China, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Peking Union Medical College, China, and Wuhan Jinyintan Hospital Wuhan Institute of Virology, Chinese Academy of Sciences, China). We acknowledge Professor Yong-Zhen Zhang, Shanghai Public Health Clinical Center & School of Public Health, Fudan University, Shanghai, China for release of another sequence (MN908947).

We use the term "SARS-related Coronavirus" to include the SARS virus as well as the clade of betacoronaviruses known to be associated with (mainly) rhinolophid bats across the Palearctic. The latest taxonomy classifies these viruses in a subgenus termed *Sarbecovirus*.

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Background
We used known SARS- and SARS-related coronaviruses (bat viruses from our own studies as well as literature sources) to generate a non-redundant alignment (excerpts shown in Annex). We designed candidate diagnostic RT-PCR assays before release of the first sequence of 2019-nCoV. Upon sequence release, the following assays were selected based on their matching to 2019-nCoV as per inspection of the sequence alignment and initial evaluation (Figures 1 and 2).

All assays can use SARS-CoV genomic RNA as positive control. Synthetic control RNA for 2019-nCoV E gene assay is available via EVAg. Synthetic control for 2019-nCoV RdRp is expected to be available via EVAg from Jan 21st onward.

First line screening assay: E gene assay
Confirmatory assay: RdRp gene assay

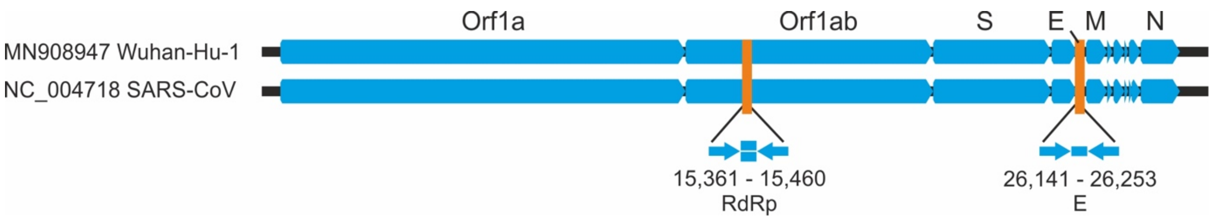


Figure 1 relative positions of amplicon targets on SARS-CoV an 2019-nCoV genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV, NC_004718.

Materials and assay formulation

Clinical samples and CoV cell culture supernatants
Respiratory samples were obtained during 2019 from patients hospitalized at Charité medical center and tested by the NxTAG® Respiratory Pathogen Panel (Luminex) or in cases of MERS-CoV by the MERS-CoV upE assay as published before (1).

Cell culture supernatants from typed coronaviruses were available at our research and clinical laboratories. The typed avian influenza virus RNA (H5N1) was obtained from the German Society for Promotion of Quality Assurance in Medical Laboratories (INSTAND) proficiency testing panels. RNA was extracted from clinical samples by using the MagNA Pure 96 system (Roche) and from cell culture supernatants by the viral RNA mini kit (Qiagen).

Assay design
For oligonucleotide design and in-silico evaluation we downloaded all complete and partial (if >400 nucleotides) SARS-related virus sequences available at GenBank by January 1st, 2020. The list (n=729 entries) was manually checked and artificial sequences (lab-derived, synthetic etc.), as well as sequence duplicates removed, resulting in a final list of 375

sequences. These sequences were aligned and the alignment used for assay design. The alignment was later complemented by sequences released from the Wuhan cluster. All presently release sequences match the amplicons (Figure 2). An overview of oligonucleotide binding sites in all unique sequences of bat-associated SARS-related viruses is shown in the appendix.

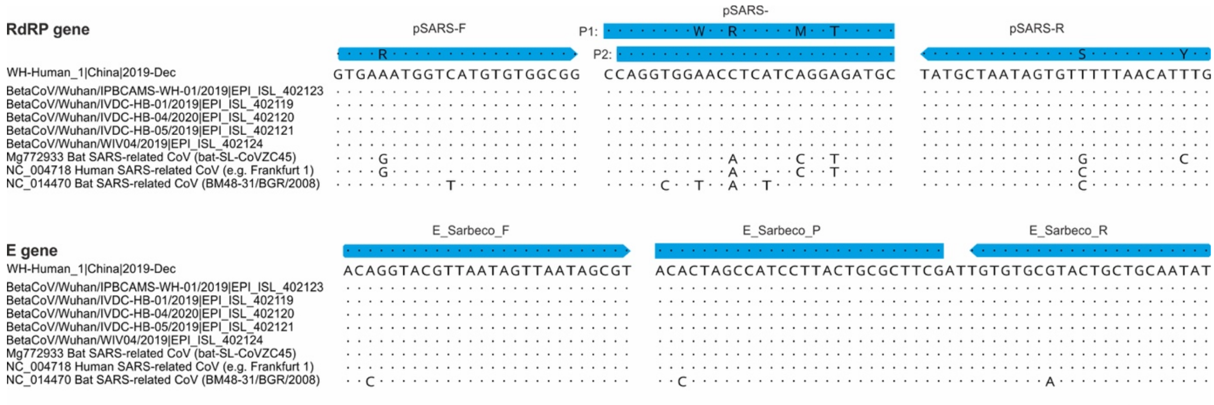


Figure 2 Partial alignments of oligonucleotide binding regions. Panels show six available sequences of 2019-nCoV, aligned to the corresponding partial sequences of SARS-CoV strain Frankfurt 1, which can be used as a positive control for all three RT-PCR assays. The alignment also contains the most closely-related bat virus (Bat SARS-related CoV isolate bat-SL-CoVZC45, GenBank Acc.No. MG772933.1) as well as the most distant member within the SARS-related bat CoV clade, detected in Bulgaria (GenBank Acc. No. NC_014470). Dots represent identical nucleotides compared to sequence Wuhan-Hu 1. Substitutions are specified. More comprehensive alignments in the Appendix.

Real-time reverse-transcription polymerase chain reaction

All assays used the same conditions. Primer and probe sequences, as well as optimized concentrations are shown in Table 1. A 25-µl reaction was set up containing 5 µl of RNA, 12.5 µl of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each deoxyribonucleotide triphosphates (dNTP) and 3.2 mM magnesium sulfate), 1 µl of reverse transcriptase/Taq mixture from the kit, 0.4 µl of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), and 1 µg of nonacetylated bovine serum albumin (Roche). All oligonucleotides were synthesised and provided by Tib-Molbiol, Berlin. Thermal cycling was performed at 55°C for 10 min for reverse transcription, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 30 s.

Table 1. Primers and probes
Optimized concentrations are mol per liter of final reaction mix.
(e.g., 1.5 microliters of a 10 micromolar (uM) primer stock solution per 25 microliter (ul) total reaction volume yields a final concentration of 600 nanomol per liter (nM) as indicated in the table)
-note that standard, non-optimized reaction conditions as indicated by suppliers of one-step RT-PCR kits will generally yield sufficient sensitivity-

Assay/ Use	Oligonucleotide ID	Sequence (5'–3')	Comment
RdRP gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	use 600 nM per reaction
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	use 800 nM per reaction
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe, will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs use 100 nM per reaction and mix with P2
E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	use 400 nM per reaction
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	use 400 nM per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	use 200 nM per reaction

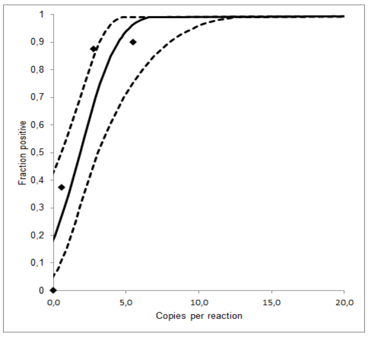
W is A/T; R is G/A; M is A/C ; FAM, 6-carboxyfluorescein; BBQ, blackberry quencher

Technical sensitivity testing

Preliminary assessment of analytical sensitivity for RdRp assay.

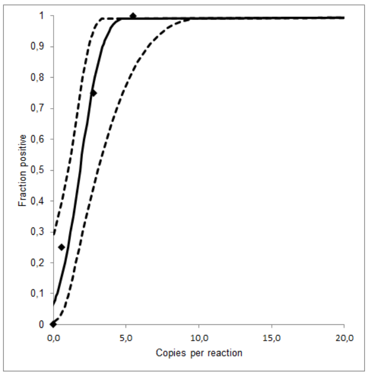
We tested purified cell culture supernatant containing SARS-CoV strain Frankfurt-1 virions grown on Vero cells, and quantified by real-time RT-PCR assay as described in Drosten et al. (2) using a specific *in-vitro* transcribed RNA quantification standard. The results are shown in Figure 3. All assays are highly sensitive.

A



A. First line assay: E gene
Technical limit of detection (LOD) = 5.2 RNA copies/reaction, at 95% hit rate; 95% CI: 3.7-9.6 RNA copies/reaction.

B



B. Confirmatory assay: RdRP gene
Technical LOD = 3.8 RNA copies/reaction, at 95% hit rate; 95% CI: 2.7-7.6 RNA copies/reaction.

Figure 3. A, E-gene assay, B, RdRp gene assay. X-axis shows input RNA copies per reaction. Y-axis shows positive results in all parallel reactions performed, squares are experimental data points resulting from replicate testing of given concentrations (x-axis) in parallels assays (8 replicate reactions per datum point). The inner line is a probit curve (dose-response rule). The outer dotted lines are 95% confidence intervals.

RdRp assay sensitivity with single probe application using the assay variant that only contains the 2019-nCoV specific probe.

SARS 2019-nCoV

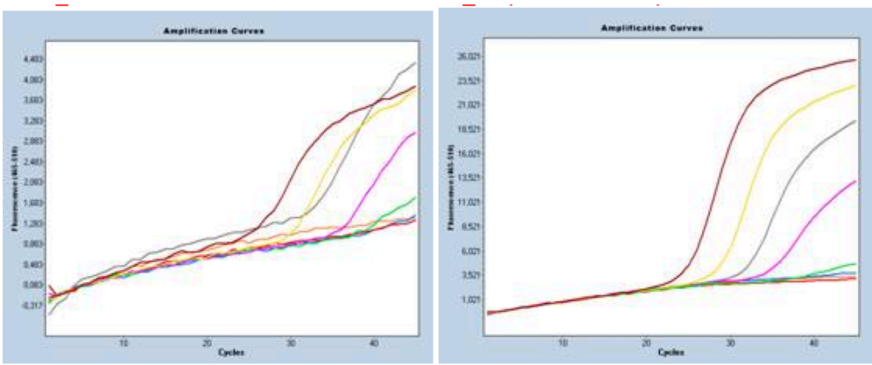


Figure 4. Preliminary experiment comparing single probe assay for SARS-CoV (probe RdRP_SARSr-P1, left panel) with single probe assay for 2019-nCoV (probe RdRP_SARSr-P2, right panel). Note that the fluorescent signal in these assays is suboptimal due to the use of PCR-generated targets.

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Breadth of detection

To show that the assays will detect other bat-associated SARS-related viruses, we tested bat-derived fecal samples available from Drexler et al., (3) und Muth et al., (4) using the novel assays.

KC633203, Betacoronavirus BtCoV/Rhi_eur/BB98-98/BGR/2008
KC633204, Betacoronavirus BtCoV/Rhi_eur/BB98-92/BGR/2008
KC633201, Betacoronavirus BtCoV/Rhi_bla/BB98-22/BGR/2008
GU190221 Betacoronavirus Bat coronavirus BR98-19/BGR/2008
GU190222 Betacoronavirus Bat coronavirus BM98-01/BGR/2008
GU190223, Betacoronavirus Bat coronavirus BM98-13/BGR/2008

All samples were successfully tested positive by the E gene assay. Detection of these relatively distant members of the SARS-related CoV clade suggests that all Asian viruses are likely to be detected.

Specificity testing

1. Chemical stability
To exclude non-specific reactivity of oligonucleotides among each other, both assays were tested 40 times in parallel with water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected.

2. Cross-reactivity with other coronaviruses
Cell culture supernatants containing human coronaviruses (HCoV)-229E, -NL63, -OC43, and -HKU1 as well as MERS-CoV were tested in all three assays (Table 2). For the non-cultivable HCoV-HKU1, supernatant from human airway culture was used. Virus RNA concentration in all samples was determined by specific real-time RT-PCRs and in-vitro transcribed RNA standards designed for absolute viral load quantification.

Table 2. Cell-culture supernatants tested by all assays

Cell culture supernatants	Tested concentration	Result
Alphacoronaviruses		
Human coronavirus NL63	4x10^9 RNA copies/ml	No reactivity with any of three assays
Human coronavirus 229E	3x10^9 RNA copies/ml	No reactivity with any of three assays
Betacoronaviruses		
Betacoronavirus 1 (strain HCoV-OC43)	1x10^10 RNA copies/ml	No reactivity with any of three assays
Human coronavirus HKU1 (HCOV-HKU1)	1x10^5 RNA copies /ml	No reactivity with any of three assays
Middle East respiratory syndrome-related coronavirus (strain EMC/2012)	1x10^8 RNA copies/ml	No reactivity with any of three assays

3. Tests of human clinical samples previously tested to contain respiratory viruses

Both assays were applied on human clinical samples from our own diagnostic services, previously tested positive for the viruses listed in Table 3. All tests returned negative results.

Table 3. Tests of known respiratory viruses and bacteria in clinical samples

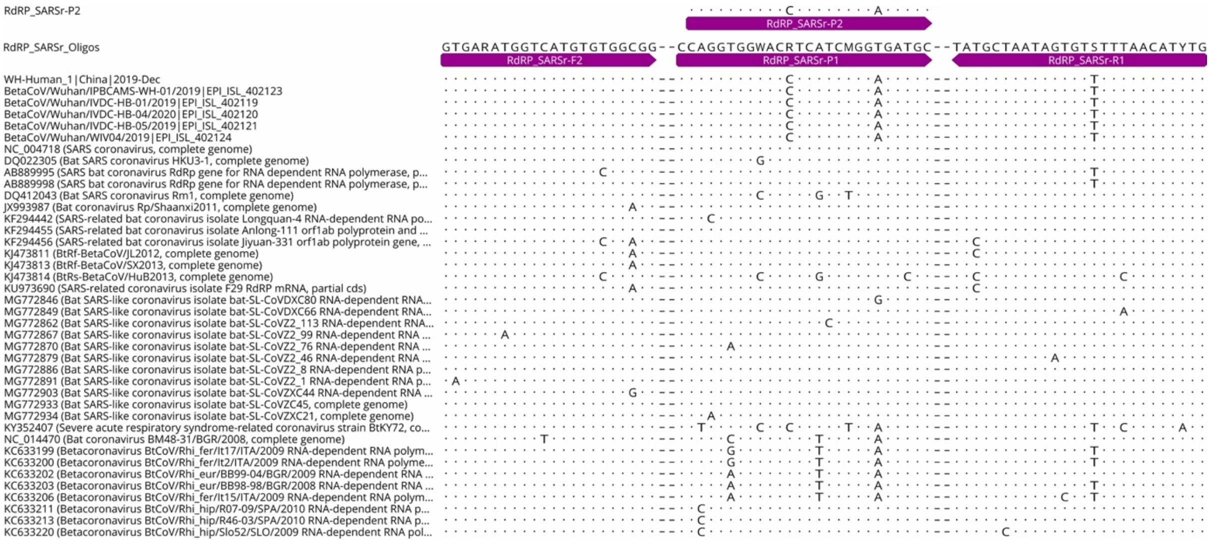
Clinical samples with known viruses	**Number of samples tested**
HCoV-HKU1	2
HCoV-OC43	5
HCoV-NL63	5
HCoV-229E	5
MERS-CoV	5
Influenza A (H1N1/09)	6
Influenza A (H3N2)	5
Influenza A(H5N1)	1
Influenza B	3
Rhinovirus/Enterovirus	3
Respiratory syncytial virus (A/B)	6
Parainfluenza 1 virus	3
Parainfluenza 2 virus	3
Parainfluenza 3 virus	3
Parainfluenza A or -B virus	5
Human metapneumovirus	3
Adenovirus	3
Human Bocavirus	3
Legionella spp.	3
Mycoplasma spp.	3
Total clinical samples	75

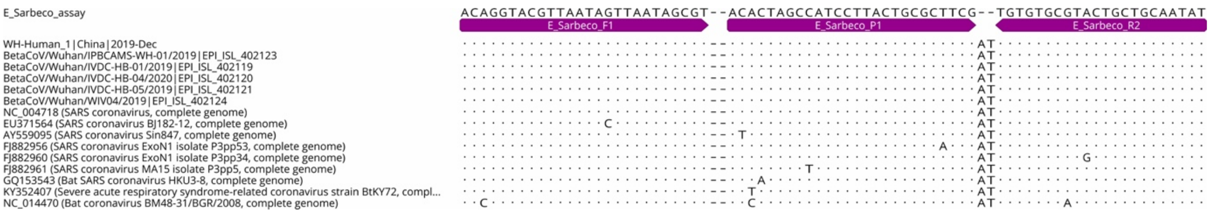
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References

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2. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1967-76.
3. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, et al. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. J Virol. 2010;84(21):11336-49.
4. Muth D, Corman VM, Roth H, Binger T, Dijkman R, Gottula LT, et al. Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. Sci Rep. 2018;8(1):15177.

Annex:





Annex figure. Non-redundant alignments of SARS-related CoVs focused on oligonucleotide binding sites of all assays (top to bottom: RdRp, E, N). Viruses not present in these alignments have been removed because their binding sites are 100% identical to one of the members of the alignment. (“--“) means sequence gaps not covered by oligonucleotides. Note that these alignments contain only one sequence of 2019-nCoV while Figure 2 above contains all presently released sequences. We will fuse this into one figure.

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Workflow Protocol

1. First line screening assay

E assay:

MasterMix:	**Per reaction**	
H_2O (RNAse free)	2.6 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer E_Sarbeco_F1 (10 µM stock solution)	1 µl	ACAGGTACGTTAATAGTTAATAGCGT
Primer E_Sarbeco_R2 (10 µM stock solution)	1 µl	ATATTGCAGCAGTACGCACACA
Probe E_Sarbeco_P1 (10 µM stock solution)	0.5 µl	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C 10’
94°C 3’
94°C 15’’ |
58°C 30’’ | 45x

If assay No 1 is positive, continue to assay No 2.

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2. Confirmatory assay

RdRp assay:

MasterMix:	**Per reaction**	
H_2O (RNAse free)	0.6 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer RdRP_SARSr-F2 (10 µM stock solution)	1.5 µl	GTGARATGGTCATGTGTGGCGG
Primer RdRP_SARSr-R1 (10 µM stock solution)	2 µl	CARATGTTAAASACACTATTAGCATA
Probe RdRP_SARSr-P1 (10 µM stock solution)	0.5 µl	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ
Probe RdRP_SARSr-P2 (10 µM stock solution)	0.5 µl	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C	10’	
94°C	3’	
94°C	15’’	
58°C	30’’	45x

If assay No 2 is positive, continue to assay No 3.

3. Discrimatory assay

RdRp assay:

MasterMix:	**Per reaction**	
H_2O (RNAse free)	1.1 µl	
2x Reaction mix*	12.5 µl	
$MgSO_4$(50mM)	0.4 µl	
BSA (1 mg/ml)**	1 µl	
Primer RdRP_SARSr-F2 (10 µM stock solution)	1.5 µl	GTGARATGGTCATGTGTGGCGG
Primer RdRP_SARSr-R1 (10 µM stock solution)	2 µl	CARATGTTAAASACACTATTAGCATA
Probe RdRP_SARSr-P2 (10 µM stock solution)	0.5 µl	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
SSIII/Taq EnzymeMix*	1 µl	
Total reaction mix	20 µl	
Template RNA, add	5 µl	
Total volume	25 µl	

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C	10’	
94°C	3’	
94°C	15’’	45x
58°C	30’’	

Assay No 3 is specific for 2019-nCoV

Note: Other generic real-time RT-PCR reagents can be used for all assays. In this case, use oligonucleotides at concentrations indicated. If using Light Cycler instrument with glass capillaries, use Light Cycler-specific reagents or add BSA as indicated in the detailed documentation above.